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INTRODUCTION

This report serves as a progress report on the second year of my training grant. I have completed my fifth year as a graduate student in Dartmouth Medical School's Department of Pharmacology and Toxicology, part of the Program in Experimental Molecular Medicine (PEMM). Dr. James DiRenzo serves as my mentor and chair of my thesis committee.

BODY

Training Tasks

I completed all required coursework and passed the qualifying exam, consisting of a written grant proposal and its oral defense. Senior members of the DiRenzo lab trained me in mouse handling, histology and tumor pathology. I was also trained to isolate mammary epithelial cell populations by fluorescence activated cell sorting (FACS), and have developed some of my own protocols for FACS-based experiments. Dr. DiRenzo has also trained me in the surgical technique of transplanting mammary epithelial cells into cleared mammary fat pads. In the past year I have also worked with our lab manager, Pratima Cherukuri, M.Sc, to develop a chromatin immunoprecipitation (ChIP) protocols to study the binding of transcription factors and transcriptional machinery at specific loci.

Meetings Attended

ISSCR 8th Annual Meeting, San Francisco, June 16-19, 2010
The Jackson Laboratory Regional Symposium for Cancer Biology, The Jackson Laboratory, Bar Harbor, September 26-27, 2010

Posters Presented

MYC oncoprotein regulates stem cell populations in both human mammary epithelial cell lines and mouse mammary glands. Presented at the ISSCR 8th Annual Meeting, June 18, 2010. Abstract included as Appendix 1, poster panels included as Appendix 2.

Research Tasks

Aim 1: Lentiviral mediated gene targeting of self-renewing and non-self-renewing mammary epithelial populations.

I made high-titer lentivirus-containing media by cotransfection of 293T cells with separate plasmids coding the viral proteins GAG and POL, plus the lentiviral expression vector pLOVE bearing either the gene for green fluorescent protein (GFP) or the N-myc oncogene. Infection efficiency near 100% was achieved in mammary epithelial cell lines by addition of polybrene to viral stocks before infection, as determined by GFP expression.

In vitro studies were done in Immortialized Mammary Epithelial Cells (IMEC), a human cell line established by my mentor James DiRenzo some years ago by telomerase-mediated immortalization of cells obtained from reduction mammoplasties. Protein expression profiles reveal IMECs to resemble basal epithelium. Functionally, they have characteristics of both stem and progenitor cells. Lentiviral expression of N-myc in IMEC cells grown in a monolayer triggered apoptosis in a large proportion of the cells; remaining cells failed to proliferate. By contrast, lentiviral expression of N-myc in IMEC cells grown in the low-binding mammosphere assay did not promote cell death; the rate of mammosphere formation from the N-myc expressing cells was comparable to the GFP control, but the spheres formed were slightly larger and had a smoother, rounder shape, suggesting an increased proportion of symmetrical mitotises.

Infection efficiency of primary mouse mammary epithelial cells was lower, but a small fraction of GFP-positive cells were clearly visible in low-binding cultures of isolated primary mammary epithelial cell populations after several days. Transplantation of lentiviral-infected primary cells did not produce epithelial outgrowths with detectible GFP expression within 10 weeks. We subsequently experimented with adenoviral infection of primary cells with an adenovirus bearing the LacZ gene, but expression of the LacZ beta-galactosidase product was not detectable in transplant recipients.

Aim 2: Transgenic targeting of oncogenic alleles into mammary stem cells.

ΔN-p63-eGFPcre and K14-eGFPcre transgenes were constructed as described in the original grant proposal. When these transgenes were transfected into mammary epithelial cell lines, limited GFP expression was observed, consistent with the concept of targeting a self-renewing, stem cell-like subpopulation. The transgenic mouse lines we created using these transgenes, by contrast, did not have a detectable subpopulation expressing GFP protein either when whole-mounted glands were observed under the fluorescence dissecting microscope, by IHC of fixed tissue, or when the dissociated epithelial cells were evaluated by flow cytometry.

Since the goal of this project was to determine whether the ΔN-p63 or K14 promoters could be used to target oncogene expression to distinct subpopulations, we proceeded to determine whether these transgenes were being transcribed in the different epithelial subpopulations. Using FACS based on surface markers CD24 and CD29, we isolated stem-cell-enriched, luminal progenitor cell and stromal cell subpopulations from transgenic and wild-type siblings. RNA was isolated from these cells and analyzed by reverse transcription-polymerase chain reaction analysis (RT-PCR). GFP mRNA was not found in ΔN-p63-eGFPcre mammary gland. In the K14-eGFPcre mouse, GFP mRNA was detected in both the stem cell and progenitor cell fractions.

Another transgene described in the original proposal, the ΔN-p63 promoter fused to a LoxP-flanked stopper sequence and cDNA for c-myc (ΔN-p63-LSL-c-myc), was tested in vitro in IMECs, because they have a uniformly high expression of the ΔN-p63 protein.

First, the transgene was transfected into IMECs, then cells with stable integration of the transgene were selected by exploiting a neomycin resistance gene. The cells were then infected with an adenovirus bearing a gene for Cre-recombinase. Successful genomic recombination was detected by polymerase chain reaction (PCR) analysis of genomic DNA. RT-PCR analysis was used to determine expression of transgenic c-myc RNA. No such RNA was detected, so that transgene was abandoned.

Seeking another method to determine the effects of c-myc expression on the mammary stem cell in vivo, we obtained an MMTV-myc transgenic mouse. The MMTV-myc mouse is a classic model of human breast cancer. MYC expression is specific to the mammary epithelium, but NOT dependent on lactation, and not during early development. Nulliparous mice have low rates of mammary tumorigenesis, but virtually 100% of parous mice develop mammary tumors. (Reviewed in Hanahan, 2007). These mice exhibit premature lactation during pregnanacy, and the involution response if the mammary gland to milk stasis is not compromised. (Blakely, 2005). Transplantation of stem cells from mature MMTV-myc mice and wild-type siblings into wild-type recipients allowed for the expression of MYC in a mitotically active mature stem cell. Analysis of the resulting grafts revealed an apparent phenotype, but an inconsistent one.

In 2010, several papers were published that together suggested an intriguing possible role for MYC in adult stem cells. First, MYC was revealed to regulate transcriptional elongation by recruiting P-TEFb to phosphorylate paused polymerases at Serine 2 of the C-Terminal Domain (CTD-S2) (Rahl, 2010). Soon after, it was convincingly demonstrated that several types of quiescent adult stem cells are deficient in terms of phosphorylation of CTD-S2 (Freter, 2010). Therefore, I hypothesize that MYC alters the transcriptional milieu of mammary stem cells by promoting transcription of a subset of genes that are normally not elongated in quiescent adult stem cells.

First, I determined that there exists a CTD-S2 phosphorylation-deficient population in normal human breast by immunofluorescence. Next, I established a model of CTD-S2 phophorylation-deficient quiescence in IMEC cells. Finally, I studied the consequences of CTD-S2 phosphorylation deficiency on the transcription of a several candidate genes, both by RT-PCR analysis and by chromatin immunoprecipitation (ChIP), using antibodies directed against RNA polymerase II. The next step will be microarray analysis. The ultimate goal of this line of inquiry will be to demonstrate a global effect of MYC on transcriptional machinery in this model of stem cell quiescence.

KEY RESEARCH ACCOMPLISHMENTS

1. The successful isolation and transplantation of an enriched population of mammary stem cells.
2. The ruling out of the K14 and ΔN-p63 promoters as efficient means of targeting specific epithelial subpopulations in transgenic mice.
3. The development of an in vitro model of mammary stem cell quiescence.

REPORTABLE OUTCOMES

There are no reportable outcomes at this time.

CONCLUSION

I have made significant progress, both toward my technical training, toward fulfilling the aims that I laid out in the proposal for this grant, and toward the goal of understanding the effects of oncogenic activity in mammary stem cells. In the next year, I hope to publish my findings, defend my thesis, and to find a post-doctoral position in a lab where I can complete my training.

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APPENDICES

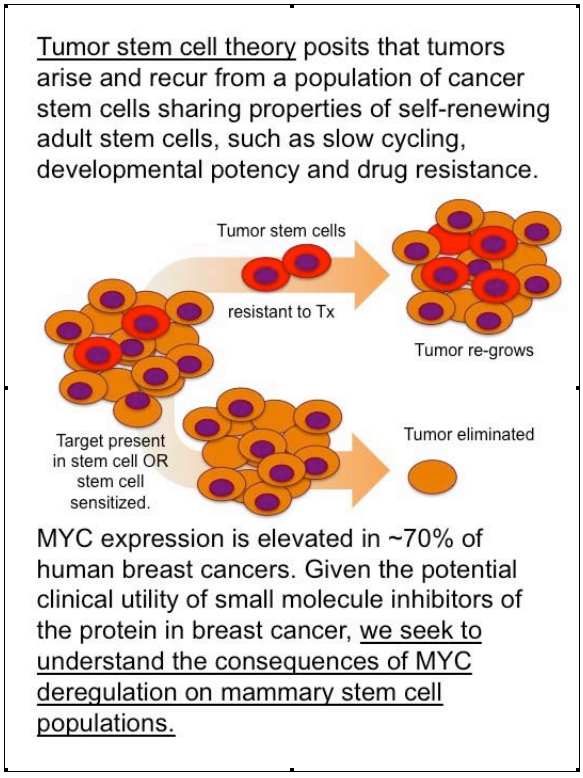
Appendix 1
Abstract from the poster entitled *MYC oncoprotein regulates stem cell populations in both human mammary epithelial cell lines and mouse mammary glands*, presented at the ISSCR 8th Annual Meeting in San Francisco on June 18, 2010.

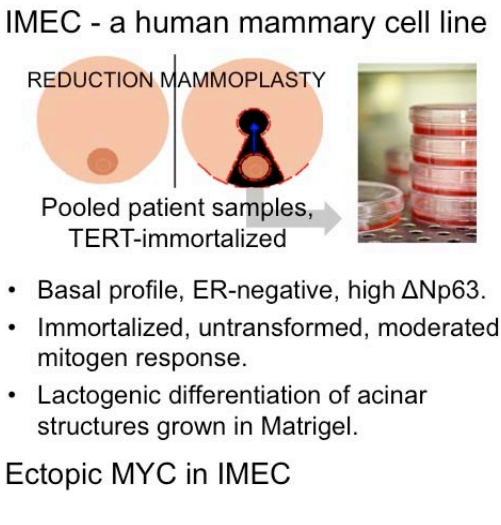
Tumor stem cell theory predicts that tumors arise and recur from a population of cancer stem cells sharing properties of self-renewing adult stem cells, including quiescence and developmental potency. Proto-oncogenic transcription factor c-Myc has documented functions in replication, growth, metabolism, differentiation, and apoptosis. Myc expression is elevated in approximately 70% of human breast cancers, and small molecule inhibitors of the Myc protein are in development. Given the potential clinical utility of these inhibitors in breast cancer, we sought to determine the effect of Myc deregulation on mammary stem cell populations. Ectopic Myc expression in hTERT immortalized human mammary epithelial cells enhances mammosphere formation, an *in vitro* assay of self-renewal potential. The MMTV-Myc mouse is a model of human breast cancer with Myc expression targeted to the mammary epithelium and high-penetrance mammary tumorigenesis following pregnancy. Premalignant abnormalities observed in nulliparous mice indicate that ectopic Myc leads to aberrant mammary regenerative cycle kinetics and aberrant mammary stem cell activity. An enriched fraction of mammary stem cells ($Lin^-/CD24^+/CD29^{high}$) from MMTV-Myc mice exhibits enhanced mammosphere formation. Syngeneic

transplantation of this population into cleared mammary fat pads produced heterogeneous structures suggesting a loss of regenerative cycle synchronization. Analysis of the phosphorylation state of ΔNp63α in a nulliparous MMTV-Myc mouse indicates high levels of ΔNp63α phosphorylation at serines 66 and 68 relative to nulliparous wild-type mice. TP63 encodes a P53-family transcription factor required for the preservation of epithelial stem cells, including mammary stem cells, and expression of the ΔNp63α isoform is limited to the $CD24^{+}/CD29^{high}$ mammary stem cell-enriched fraction. Ongoing work in our lab has correlated this phosphorylation of ΔNp63α with stem cell proliferation suggesting that immunologic detection of phospho-ΔNp63α may distinguish normal and transformed mammary stem cells. These data support the hypothesis that Myc promotes tumorigenesis in the mammary gland through activation of a mammary stem cell population.

Appendix 2

Panels from the poster entitled *MYC oncoprotein regulates stem cell populations in both human mammary epithelial cell lines and mouse mammary glands*, presented at the ISSCR 8th Annual Meeting in San Francisco on June 18, 2010.

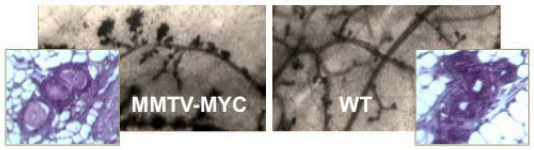




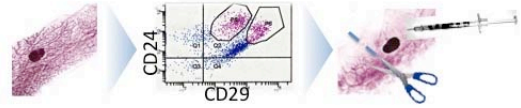
(6)

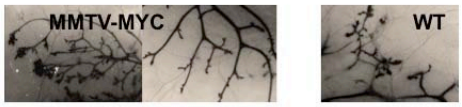
The MMTV-MYC mouse

- Ectopic MYC is mammary epithelium specific, lactation independent.
- Pregnancy ➔ premature lactation & involution, increased tumorigenesis
- Nulliparous mice exhibit alveolar formation and dilation of ducts:



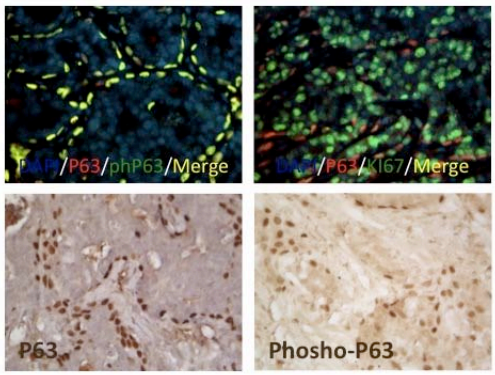
MMTV-MYC stem cell outgrowths





Asynchronous regenerative cycles suggest aberrant mammary stem cell activity.

MMTV-MYC mammary tumors



P63 is phosphorylated at pregnancy D14, a period of stem cell mitotic activity.